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Effects of Light and Food as Zeitgebers on Locomotor Activity Rhythms in the Loach, *Misgurnus anguillicaudatus*

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ABSTRACT—Four experiments were performed to examine the synchronizing effects of light and food on the locomotor activity rhythm in the loach (*Misgurnus anguillicaudatus*). In Exp. 1, food was given once a day at the scheduled time in the middle of light (L) or dark (D) phase under LD 12:12. When the fish were fed in the L phase, they showed three types of activity pattern (light-active, dark-active and light-dark-active). On the other hand, most of the fish became dark-active when they were fed in the D phase. In both conditions, the fish were entrained well to the scheduled feeding. In Exp. 2, the scheduled feeding cycle was removed from the previous Exp. 1. Under these conditions, the loach remained to show the same pattern as that in Exp. 1 or tended to become dark-active. The feeding-anticipatory activity peak gradually disappeared during these experiments. In Exp. 3, the fish were exposed to the scheduled feeding and constant darkness (DD). Since almost all fish were entrained to the scheduled feeding, scheduled feeding as well as LD cycles is effective as a zeitgeber for the locomotor activity rhythm in the loach. Under constant conditions (Fig. 4), free-running rhythms were observed, although the duration of the rhythm was short and the ratio of the individuals that showed free-running rhythms was low (7–50%). Therefore, the locomotor activity rhythm in the loach is an endogenous rhythm but the coupling between the oscillator and the locomotor activity seems to be weak and different depending on individuals.

INTRODUCTION

One of the characteristics of the circadian rhythms in fishes is variability of the rhythms compared with those in higher vertebrates [15]. The appearance of the circadian rhythms varies inter- [24] and intra-specifically [5, 7, 27], and even intra-individually [12]. Activity patterns showed diurnal, nocturnal, crepuscular and intermediate types depending on fishes [24]. In the perch (*Perca fluviatilis*), juvenile fish showed a nocturnal activity pattern but adult fish changed the activity pattern to diurnal [7]. Several activity patterns appeared simultaneously such as diurnal and nocturnal in the juvenile pink salmon (*Oncorhynchus gorbuscha*) [5] and diurnal, nocturnal, light-change-active and arrhythmic in the medaka (*Oryzias latipes*) [27]. Locomotor activity changes seasonally in the minnow (*Phoxinus phoxinus*), the sculpin (*Cottus poecilopus*) and the burbot (*Lota lota*) [12], and the medaka [31]. These reports suggest that a plastic reactivity to various zeitgebers may induce the variability of the rhythm. In order to clarify this probability, we selected two environmental factors, light and food. Light is a major environmental factor as a zeitgeber in fishes as well as in other organisms. Food has been reported to act as a zeitgeber in some fishes, such as the goldfish (*Carassius auratus*) [14] and the medaka [29].

The loach (*Misgurnus anguillicaudatus*) is one of the common freshwater fishes in Japan and inhabits paddy fields and small streams. Although there are several studies concerning the spawning behavior and season in the loach [19, 23], there is only one report by Yanagishima and Mori [30] on the locomotor activity rhythm of the loach. They reported that the fish showed the exogenously controlled nocturnal activity rhythm because the rhythmicity immediately disappeared under a constant condition. In this paper, we investigated (1) effects of single environmental factor, light (LD cycle) or food (scheduled feeding cycle), on the locomotor activity rhythm, (2) effects of the phase relationship between two environmental factors, light and food, on the rhythm, and (3) whether the locomotor activity rhythm of the loach is an endogenous circadian rhythm or not.

MATERIALS AND METHODS

Adult loaches (*Misgurnus anguillicaudatus*) including males and females of about 9–16 cm in total length were used. We caught them at a small stream along the paddy fields in Kyoto Prefecture in May, 1988, or obtained cultured fish in the Shikoku districts from a fish shop in April to October, 1987.

The fish were kept individually in a plastic water tank (31.5×17×21H cm) with sand at the bottom and placed in a bioclimatic chamber at 25±1°C at least one month before experiments. Fluorescent lamps (40 W) were used as the light source. Light intensities at the water surface were measured by a radiometer (UDT161, United Detector Technology Inc., California) and adjusted at 460–600 lux (0.26–0.39 mW/cm^2) or 5 lux (0.002 mW/cm^2) in the light (L) phase and 0 lux in the dark (D) phase. We fed about 0.5–1.0 g wet weight of live tubifexes as food by hand or an auto

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feeding instrument (SP-10A, Nippon Denshi Kagaku, Kyoto) during scheduled feeding experiments. Tubifexes with water were put in a small plastic tube and thrown into a water tank by overturning this tube at the feeding time. As a control, we gave only water using the tube for 3 days just after the scheduled feeding experiment, but none of the fish reacted to this sham-feeding regimen. Water was always aerated and filtered. The locomotor activity of the loach was measured by a pair of infrared photocells (JU-33P, -33R or PL3-E, -FL, Hokuyo Denki, Osaka) at both sides of the water tank. The light source and receiver of photocells were set at about 1 cm above the sand, since the loach is a benthic fish. Main activity including searching and feeding behavior and most swimming behavior was observed at the bottom layer (see the results of Exp. 3). When the fish interrupted the beam, it was recorded by an actograph (Fuji Denki, Tokyo) and the number of interruptions were counted by a digital data recorder (DDR3010, Sanyo Denki, Osaka). Data from the actograph were used for visual examination, and total counts per one hour from the digital data recorder were used for the statistical analyses such as χ^2-test and periodogram analysis. In these analyses, the significance level we adopted was 95% confidence limit.

In the present study, we selected four experimental conditions to examine the synchronizing effects of light-dark (LD) cycles and scheduled feeding in the locomotor activity rhythm of the loach and compared the results individually by using the same members of fish throughout these experiments. Before each experiment, loaches were subjected to constant darkness (DD) without food for at least 10 days to eliminate the after-effect of the previous condition.

Experiment 1 Locomotor activity rhythms under LD cycles and scheduled feeding.

(A) Food was given at the scheduled time (12:00) in the middle of the L phase under LD 12:12 (L: 06:00–18:00, D: 18:00–06:00; L=460–600 lux, D=0 lux) for 10 (n=6) or 15 (n=14) days.

(B) Food was given at the scheduled time (12:00) in the middle of the D phase under reversed LD 12:12 (L: 18:00–06:00, D: 06:00–18:00) for 12 (n=6) or 15 (n=14) days.

Experiment 2 Effects of LD cycles on the locomotor activity rhythms without food after Exp. 1.

(A) Fish (n=6) were placed under LD 12:12 without food after Exp. 1-A for 10 days.

(B) Fish (n=6) were placed under LD 12:12 without food after Exp. 1-B for 10 days.

Experiment 3 Effects of scheduled feeding on the locomotor activity rhythms under constant darkness (DD).

Fish (n=6) were fed once a day at the scheduled time (12:00) under DD (D=0 lux) for 11 days. In order to analyze the behavior of the loach under the scheduled feeding and constant dim light (dim LL, L=5 lux), we recorded the behavior of a female by VTR (TV camera; WV-1550, Matsushita Tsushin, Osaka, Time Lapse VTR; NV-720, Matsushita Denki, Osaka).

Experiment 4 Free-running rhythms under constant conditions after Exp. 1 and 3.

(A) Fourteen fish were subjected to DD without food after Exp. 1-A for 15 days.

(B) Fourteen fish were placed under DD without food after Exp. 1-B for 15 days.

(C) Six fish were placed under DD without food after Exp. 3 for 14 days.

All experiments were performed during November, 1987 to September, 1988.

RESULTS

Experiment 1 Locomotor activity rhythms under LD cycles and scheduled feeding.

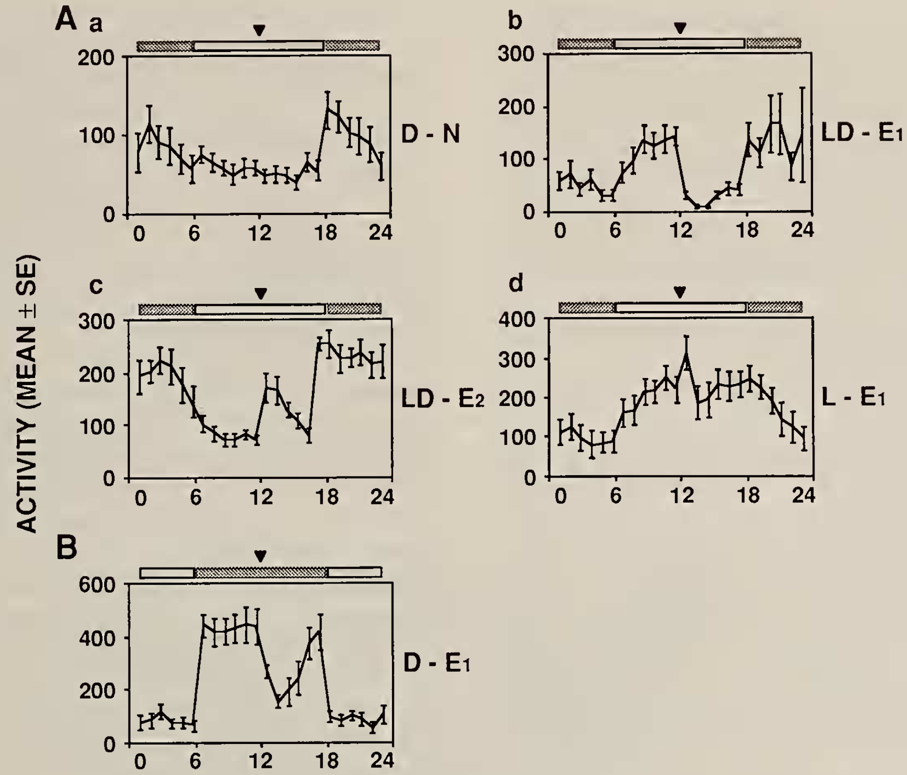
(A) Food was given at the scheduled time (12:00) in the middle of the L phase under LD 12:12.

The locomotor activity of the loach was classified into four patterns in relation to LD cycle, i.e., dark-active (nocturnal) (ex. Fig. 1A-a, B), light-dark-active (ex. Fig. 1A-b, c and Fig. 2a), light-active (diurnal) (ex. Fig. 1A-d) and arrhythmic. The term "light-dark-active" denotes that there are peaks of activity in both L and D phases. Among 20 fish used in this experiment, one fish was dark-active, 12 were light-dark-active and seven were light-active, and the difference was statistically significant ($P<0.05$, χ^2-test for one sample) (Table 1a). It took at least 3–5 days for the loaches to establish the stable locomotor activity pattern (see Fig. 2a). Four out of six light-active fish tended to change their activity patterns from dark- to light-active during this experiment. All fish excluding one dark-active fish (Fig. 1A-a) were entrained to scheduled feeding ($P<0.01$, χ^2-test for one sample), and the activity was classified into two types as follows. Type 1 (E_1): the peak of activity lasted several hours before the feeding time, which probably reflects the anticipation for food (n=11), and among these 11 fish, eight remained inactive for several hours after feeding (ex. Fig. 1A-b) and three continued to be active for several hours after feeding (ex. Fig. 1A-d). Type 2 (E_2): the activity peak lasted several hours after the feeding time without the anticipatory peak (n=8) (ex. Fig. 1A-c). When Exp. 1-A regimen was repeated after Exp. 2-A using the same individuals, a similar tendency of activity patterns was observed for both LD cycle and scheduled feeding.

(B) Food was given at the scheduled time (12:00) in the middle of the D phase under LD 12:12.

In this experiment, all fish except one light-dark-active fish showed a dark-active pattern (ex. Fig. 1B and Fig. 2b) ($P<0.01$, χ^2-test for one sample) (Table 1a). A tendency toward the change of activity pattern from light- to dark-active was observed in four dark-active fish during this experiment. All of the 19 dark-active fish were synchronous to the scheduled feeding ($P<0.01$, χ^2-test for one sample) (Table 1a). The two types of entrainment to scheduled feeding as the previous experiment were also observed in this experiment. The number of fish for type 1 and 2 was 18 (ex. Fig. 1B) and 1, respectively.

When Exp. 1-A was compared with 1-B, various activity patterns appeared under the scheduled feeding in the L phase, while the loach became exclusively dark-active under the scheduled feeding in the D phase, and the difference was highly significant ($P<0.01$, χ^2-test for multiple samples) (Table 1a). When the activity patterns were compared individually between these two conditions, three types were detected in the entrainment to LD cycles; (1) fish strongly affected by the scheduled feeding, as they tended to be light-active when they were fed in the L phase and dark-active



TIME OF DAY (HR)

FIG. 1. Examples of locomotor activity rhythms of the loach in Experiment 1. (A) Exp. 1-A (LD 12:12, Food in L). (B) Exp. 1-B (LD 12:12, Food in D). Activity patterns were represented as D, LD and L for the entrainment in relation to LD cycles, and E_1, E_2 and N for the entrainment to scheduled feeding. D: dark-active. LD: light-dark-active. L: light-active. E_1: entrained with the anticipatory activity peak (type 1, see text). E_2: entrained without the anticipatory peak (type 2, see text). N: not entrained. Horizontal bar: light condition (LD 12:12, white bar: L, dotted bar: D). Triangle: feeding time. Data were shown as mean ± standard error (SE) for 10 or 12 days.

TABLE 1. The number of individual fish for each activity pattern

(a)

Experiments	LD cycle					Scheduled feeding		
	Dark-active	Light-dark-active	Light-active	Arrhythmic		Entrained	Not entrained	
1-A (LD 12:12, Food in L)	1	12	7	0	*	19	1	**
1-B (LD 12:12, Food in D)	19	1	0	0	** †† ††	19	1	**
2-A (LD 12:12, Food removed in L)	4	0	2	0		—	—	
2-B (LD 12:12, Food removed in D)	6	0	0	0		—	—	
3 (DD, Food at 12:00)	—	—	—	—		5	1	

(b)

Experiments	Constant condition	
	Free-run	Not free-run
4-A (after LD 12:12, Food in L)	1	13
4-B (after LD 12:12, Food in D)	4	10
4-C (after DD, Food at 12:00)	3	3

*: $P<0.05$, χ^2-test for one sample **: $P<0.01$, χ^2-test for one sample
††: $P<0.01$, χ^2-test for multiple samples

when they were fed in the D phase (n=7), (2) fish affected only by LD cycles, as they were always dark-active irrelevant to the phase of feeding (n=1), and (3) the intermediate type between types (1) and (2) (n=12). In the case of entrainment to scheduled feeding, there were also three types, (1) fish entrained with an anticipatory activity peak (type 1) irrelevant to the phase of feeding (n=11), (2) fish changed their activity patterns to type 1 when they were fed in the D phase (n=7), and (3) fish did not show the anticipatory activity peak (n=2).

Experiment 2 Effects of LD cycles on the locomotor activity rhythms without food after Exp. 1.

(A) Fish were placed under LD 12:12 without food after Exp. 1-A.

In six fish observed, four were dark-active (ex. Fig. 2a) and two were light-active (Table 1a). Under LD 12:12 and the scheduled feeding at the L phase in Exp. 1-A, all of the four dark-active fish showed light-dark-active pattern, while two light-active fish were light-dark- or light-active. A significant reduction in the amount of activity was observed in the two light-active fish (ex. Fig. 2a) and one dark-active fish in a few days after the beginning of this experiment probably because of starvation. The anticipatory activity peak observed before the feeding time disappeared within a few days.

(B) Fish were placed under LD 12:12 without food after Exp. 1-B.

In this experiment, all six fish were dark-active (ex. Fig. 2b) (Table 1a). They were dark-active in the previous Exp. 1-B. Three of them decreased the amount of activity in a few days.

When the results of Exp. 2 was compared with that of Exp. 1, in five fish that were light-dark-active under the scheduled feeding in the L phase (Exp. 1-A), four fish changed their activity patterns to dark-active, and only one fish changed to light-active. One fish that were light-active in Exp. 1-A kept the same pattern. The difference between Exp. 1-A and 2-A was highly significant ($P<0.01$, χ^2-test for multiple samples) (Table 1a). On the other hand, the dark-active pattern in all of the six fish under the scheduled feeding in the D phase (Exp. 1-B) was maintained in the condition without food. Therefore, the loach is mainly a nocturnal species and the activity peak in the L phase seems to depend on the scheduled feeding in the L phase.

Experiment 3 Effects of scheduled feeding on the locomotor activity rhythms under constant darkness (DD).

Five out of six fish were considered to be entrained by the scheduled feeding (Table 1a). They showed a peak of anticipatory activity prior to the feeding time (type 1) (Fig. 3). The amount of activity reduced just after the feeding time and increased gradually until the next feeding time.

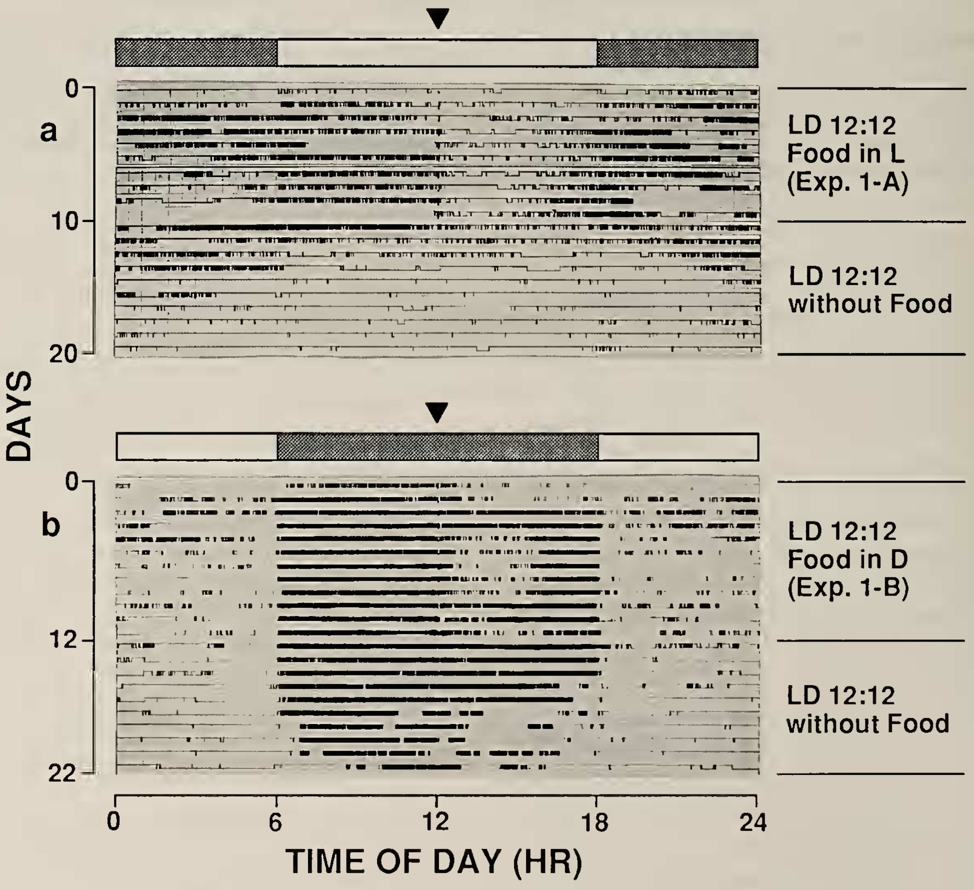


Fig. 2. Examples of locomotor activity rhythms of the loach in Experiment 2 (LD 12:12, without Food). (a) Exp. 2-A was performed after Exp. 1-A (LD 12:12, Food in L). (b) Exp. 2-B was performed after Exp. 1-B (LD 12:12, Food in D). Horizontal bar: light condition (LD 12:12).

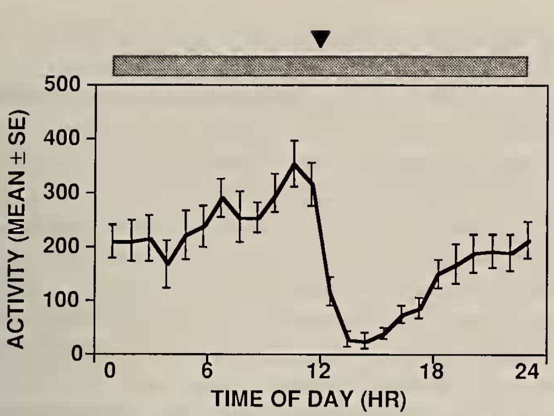


FIG. 3. An example of locomotor activity rhythms of the loach in Experiment 3 (DD, Food at 12:00). Horizontal bar: light condition (DD). Triangle: feeding time. Data were shown as mean ± SE for 11 days.

We recorded and analyzed the behavior of feeding-entrained activity pattern of a female loach under dim LL by a VTR. This female loach showed the type 1 in the feeding-entrained activity pattern. We divided the behavior of the loach into searching and feeding behavior, and other swimming behaviors. Feeding crawl specified as crawling exploration with plowing, plowing ahead and gulping (including dig and twist) was regarded as searching and feeding behavior [28]. From the results observed every one hour, only swimming behavior at the upper and/or the bottom layer was observed till 16:00 (two hours before the scheduled feeding time). Searching behavior and rest were added to swimming at 16:00–17:00. Swimming at the upper layer decreased and searching behavior relatively increased, and swimming near the place where the fish was always fed was frequently

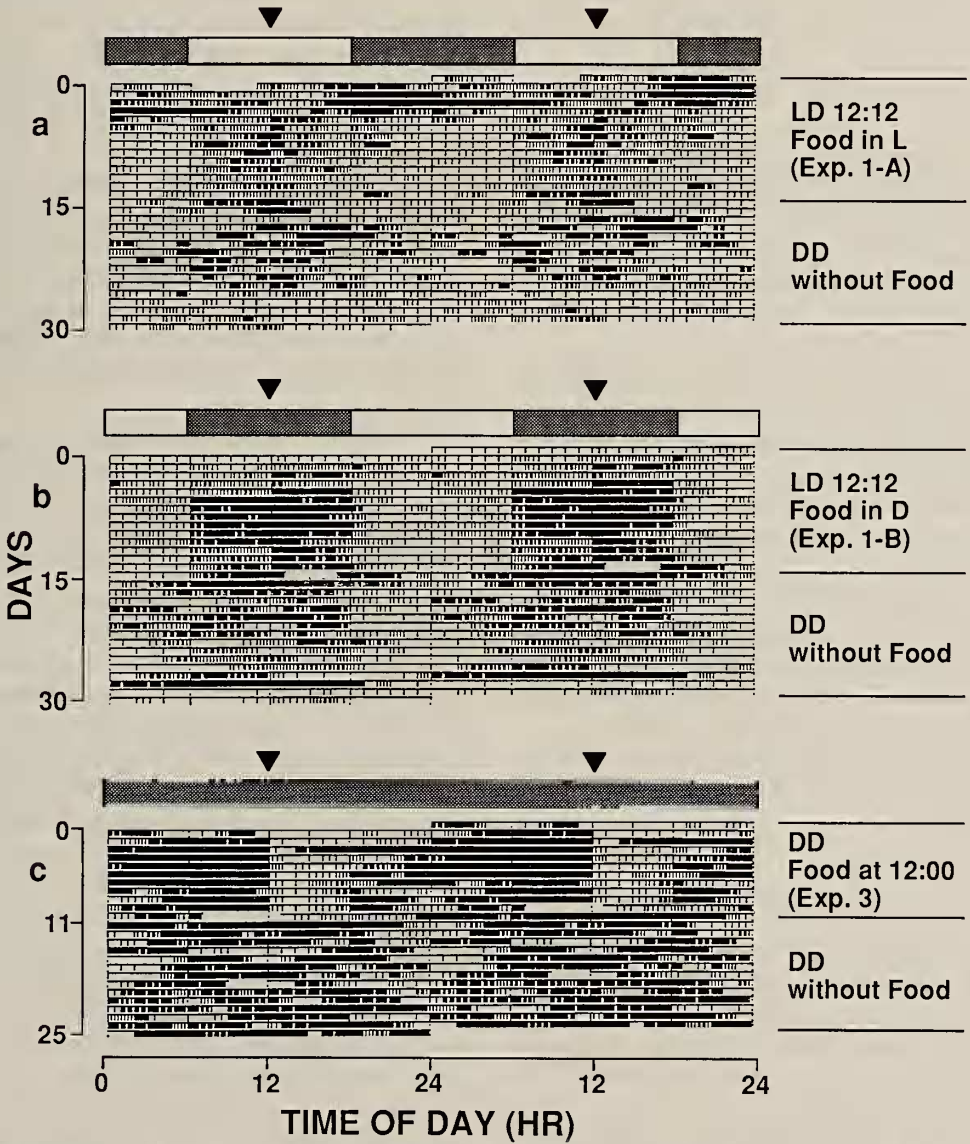


FIG. 4. Free-running locomotor activity rhythms of the loach in Experiment 4 (DD, without Food). (a) Exp. 4-A was performed after Exp. 1-A (LD 12:12, Food in L). Free-running period (τ)=23.5 hr. (b) Exp. 4-B was performed after Exp. 1-B (LD 12:12, Food in D). τ=24.1 hr. (c) Exp. 4-C was performed after Exp. 3 (DD, Food at 12:00). τ=24.7 hr. Data were double-plotted.

observed at 17:00–18:00. When the food was given at 18:00, the fish fed it within a few minutes and gradually became inactive. Whenever the fish was active, it was usually at the bottom layer. There were swimming and rest at the bottom from 19:00 to 22:00. Swimming at the upper layer appeared at 22:00–23:00 and it was increased at 23:00–0:00. This series of behavior corresponded to the feeding-entrained activity pattern of the female loach recorded by the actograph. In this experiment, it was shown that the scheduled feeding could induce the locomotor activity rhythm of the loach.

Experiment 4 Free-running rhythms under constant conditions after Exp. 1 and 3.

(A) Fish were subjected to DD without food after Exp. 1-A.

Only one of the 14 fish showed free-running rhythms for about 15 days (τ=23.5 hr) (Fig. 4a, Table 1b). This fish was light-active and entrained to scheduled feeding as type 1 in the Exp. 1-A.

(B) Fish were placed under DD without food after Exp. 1-B.

Four of the 14 fish showed free-running rhythms for about 7–13 days. One of them showed shorter free-running period than 24.0 hr (τ=22.0 hr) and three fish showed longer free-running periods than 24.0 hr (τ=24.1, 24.1 and 25.6 hr) (Fig. 4b, Table 1b). All of these fish were dark-active and entrained to scheduled feeding as type 1 in the previous Exp. 1-B.

(C) Fish were placed under DD without food after Exp. 3.

Three of the six fish showed free-running rhythms for about 5–9 days. One fish showed the free-running period of 22.1–23.7 hr and two fish showed free-running periods of 24.7 (Fig. 4c) and 28.2 hr (Table 1b). The fish with τ=28.2 hr showed free-running rhythms throughout Exp. 4-A to C. The free-running period in Exp. 4-A and B was 23.5 and 22.0 hr, respectively. Two fish with longer free-running period than 24 hr were entrained to scheduled feeding as type 1 in the previous Exp. 3 and one fish with shorter free-running period than 24 hr was not entrained to scheduled feeding.

Since free-running rhythms were observed after entrainment to the scheduled feeding, feeding can be considered as a zeitgeber in the loach.

DISCUSSION

Reports on the effect of scheduled feeding cycles on the locomotor activity rhythm have been increasing. In the goldfish (*Carassius auratus*), the scheduled feeding with LD cycle affected the locomotor activity rhythm, growth rate, and serum-cortisol and -thyroxine concentrations [14, 21]. In the medaka (*Oryzias latipes*), scheduled feeding cycles had different influences on the different types of behavior, that is, the agonistic behavior was entrained to the feeding cycle, but the egg laying and courtship behavior were entrained rather to LD cycles than to feeding cycles [29]. In the mudskipper (*Periophthalmus cantonensis*), the locomotor activity rhythm could be entrained to the 12-hour feeding cycle [13]. The channel catfish (*Ictalurus punctatus*) showed subjective feeding time under ad-lib feeding regimen [17]. In contrast, the scheduled feeding did not affect the locomotor activity rhythm in the blenny (*Blennius pholis*) [4].

Feeding both in the L phase and in the D phase could entrain the activity rhythm of the loach (Exp. 1). The activity pattern varied widely when the fish were fed in the L phase, but they were consistently dark-active when they were fed in the D phase. In Exp. 2, the activity patterns tended to change to dark-active under the regimen without food. Therefore, fundamentally the loach seems to be a nocturnal species. Benthic fishes, such as the eel, catfish and loach, have been considered as nocturnal or light-dark-active species because benthic fish approach to their food horizontally and rely on not only vision but also other senses to find food [20]. However, since cone cells and cone visual pigments (iodopsin-like substances) existed in the retina of the loach [11], they seem to have an ability to be active during daytime. In the field, probably the availability of food for the loach do not change diurnally because the loach is an omnivorous detritus feeder. In the present study, however, loaches were entrained to the daily feeding cycle, although food (tubifexes) was not taken away, and thus, the fish could feed these tubifexes alive in the bottom sand whenever they want, and this was supposed to provide a weaker influence on the entrainment than the food-removed regimen. Thus, the importance of feeding in the circadian structure of the loach should not be neglected.

Davis and Bardach [3] indicated the importance of the anticipatory activity peak that appeared prior to the scheduled feeding time. This activity peak was also mentioned by Aschoff [1], in which it was suggested to appear under both LD cycles and constant conditions. In the present study, the loach showed the anticipatory activity peak in both LD cycle and constant darkness (DD).

The loach showed conspicuous resting periods of several hours after the feeding time. There were no reports about this type of resting period in the study of the feeding-entrained rhythm. Thus, this resting period may be unique to the locomotor activity rhythm of the loach entrained to the feeding time. The reason why the loach needs this period may be due to the fact that they are benthic fish and they have to spend many hours to digest food [26].

It has been suggested in fishes that free-running rhythms are labile and do not last for a long time [15, 18], and the ratio of individuals with free-running rhythms is lower than those of higher vertebrates, although there are some exceptions such as the lake chub (*Couesius plumbeus*) [9], the goldfish [10], two species of the hagfish (*Eptatretus burgeri, Paramyxine atami*) [8, 16] and the catfish (*Silurus asotus*) [25]. In the loach, free-running rhythms lasted for 5–15 days and thus, the locomotor activity rhythm of the loach is an endogenous circadian rhythm. However, the ratio of individuals which showed free-running rhythms was low and varied from 7 to

50% depending on experiments, and the rhythm persisted only for short periods. This indicates that the extent of coupling between the oscillator and the locomotor activity seems to be weak and might differ depending on individuals.

Since the periodic feeding in the rat caused to uncouple the feeding-anticipatory peak from the component of free-running rhythm [2, 6, 22], it is suggested that the feeding-entrained oscillator is different from the LD cycle-entrained oscillator. The SCN-lesioned animal that showed arrhythmic locomotor activity also showed the anticipatory activity prior to feeding [2, 22]. Different oscillator systems for feeding and LD cycles might also exist in the loach, because the uncoupled anticipatory peak was observed and gradually disappeared in Exp. 1-A and 2-A.

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